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Cripto is a growth factor that is important in breast cancer, leading to increases in cell proliferation and to increased survival of cells. Specific receptors for this factor have not been defined for breast cells but there is evidence from published work that Cripto acts as a co-factor for the Nodal factor, previously thought to be present and active only in early embryonic development. This work will define the importance of this route of Cripto signaling in breast cells compared with the other known route involving Ras and the MAPK/Erk pathway. A number of possible ways that Cripto could effect a proliferative signal to breast cells has been described by the PI in the following review article: Eileen D. Adamson, Gabriella Minchiotti, and David. S. Salomon, (2002) CRIPTO: A Tumor Growth Factor and More, *J Cell Physiol.* 190, 267-278. The experimental studies for exploring the activation mechanism of breast cancer cells by Cripto is underway with the aim of making peptides that block Cripto and its tumorigenic effects.

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INTRODUCTION

Progress has been limited by the difficulties in hiring a suitable person to carry out the main experimental procedures. Happily, I have now appointed Dr Min Li who will start work here at the end of July. He is well qualified for the work and at his interview had some ideas about how Cripto might act on cells.

I have confidence that we can catch up with the program summarized in the Statement of Work in due course. However, according to the length of the delay, we will inevitably be behind schedule by one year. Therefore, I expect to request, at the end of the project in June 2004, that we be allowed to extend the time by one full year, without further funds. It is important work and important to me that we can produce what we set out to do.

BODY OF REPORT

Cripto is a growth factor that is important in breast cancer, leading to increases in cell proliferation and to increased survival of cells. Specific receptors for this factor have not been defined for breast cells but there is evidence from published work that Cripto acts as a co-factor for the Nodal factor, previously thought to be present and active only in early embryonic development. This work will define the importance of this route of Cripto signaling in breast cells compared with the other known route involving Ras and the MAPK/Erk pathway. A number of possible ways that Cripto could effect a proliferative signal to breast cells has been described by the PI in the following review article: Eileen D. Adamson, Gabriella Minchiotti, and David. S. Salomon, (2002) CRIPTO: ATumor Growth Factor and More, *J Cell Physiol.* 190, 267-278. The experimental studies for exploring the mechanism of activation of breast cancer cells by Cripto is underway with the aim of making peptides that block the activity of Cripto and its tumorigenic effects.

KEY RESEARCH ACCOMPLISHMENTS

- Written a review of the current knowledge on the effects and mechanisms of action of Cripto as a growth factor in embryonic and breast cancer cells. A copy is attached in the Appendix.
- Despite the delay in hiring a post-doctoral Fellow to work on this project, a suitable person has now been hired, his biosketch is attached in the Appendix.

REPORTABLE OUTCOMES

A Review Article.

Eileen D. Adamson, Gabriella Minchiotti, and David. S. Salomon, (2002) CRIPTO: A Tumor Growth Factor and More, *J Cell Physiol.* 190, 267-278.

APPENDIX

1. Review Article
2. Biosketch for Dr Min Li

Appendix E

Biographical Sketches

Provide the following information for the key personnel listed on page 1 of the Detailed Cost Estimate form (see Appendix F) for the initial budget period.

NAME MIN LI	POSITION TITLE POSTDOCTORAL ASSOCIATE

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

INSTITUTION AND LOCATION	DEGREE (IF APPLICABLE)	YEAR(S)	FIELD OF STUDY
Harbin Medical University, Harbin, China	Bachelor of Medicine	1987-1992	medicine
Harbin Medical University, Harbin, China	Master of Medicine	1992-1995	medicine
Toyoma Medical Pharmaceutical University, Toyoma, Japan	Exchange Student	1999-2000	
Peking University, Beijing, China	Ph.D.	1998-2001	molecular toxicology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past 3 years and representative earlier publications pertinent to this application. PAGE LIMITATIONS APPLY. DO NOT EXCEED THREE PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INVESTIGATOR.

PROFESSIONAL EXPERIENCE

1995-1998 Clinical Pharmacological Base Of Health Ministry Of China, Harbin Medical University, Harbin, China
Pharmacist in charge. Research in clinical pharmacology and toxicology, pharmacokinetics

2001-2002 Department Of Toxicology, Health Science Center, Peking University Beijing, China
Lecturer

2002-present Molecular and Enviromental Toxicology Center in University Of Wisconsin, Madison, U.S.A.
Post-Doctor

AWARDS AND HONORS

1. Excellent graduate in Peking University, July 2001.
2. Excellent paper award in Peking University, 2001.
3. Excellent student scholarship granted by National Education Ministry, Nov. 2000.
4. Nishiyama Award for excellent young scientist provided by Toyama Medical and Pharmaceutical University. Japan, May 2000.

PUBLICATIONS

1. Min Li, Takashi Kondo, Qing-Li Zhao, Fu-Jun Li, Kiyoshi Tanabe, Yoko Arai, Zong-Can Zhou, and

Minoru Kasuya. Cadmium induces apoptosis of U937 cell lines through Ca2+-calpain dependent and mitochondria-caspase dependent pathways. *J. Biol. Chem.* 2000; 275: 39702-39709.

2. Min li, Hui-Fang Gan. Spindle checkpoint and carcinogenesis. Carcinogenesis, Teratogenesis and Mutagenesis (Chinese) 1999; 11:145-147.
3. Min Li, Zong-Can Zhoul. Protein-chip. *Science of Life* (Chinese) 2001, 21: 156-157
4. Min Li, Tian Xia, Chun-Sun Jiang, Lin-Jiang Li, Juan-Ling Fu and Zong-Can Zhou. Cadmium directly induced the opening of membrane permeability pore of mitochondria which possibly involved in cadmium-triggered apoptosis. *Toxicology* (In Press, 2002)
5. Fu-Jun Li, Takashi Kondo, Qing-Li Zhao, Kiyoshi Tanabe, Ryohei Ogawa, Min Li, Yoko Arai. Enhancement of hyperthermia-induced apoptosis by a free radical initiator, 2,2'-azobis (2-amidinopropane) dihydrochloride, in human histiocytic lymphoma U937 cells. *Free Radic Res.* 2001, 35:281-99.
6. Yoko Arai, Takashi Kondo, Kiyoshi Tanabe, Qing-Li Zhao, Fu-Jun Li, Ryohei Ogawa, Min Li, and Minoru Kasuya. Enhancement of hyperthermia-induced apoptosis by local anesthetics on human hystiocytic Lymphoma U937 cells. *J. Biol. Chem.* (in Press: published February 22, 2002)

JOURNAL OF CELLULAR PHYSIOLOGY 190:267–278 (2002)
DOI 10.1002/jcp.10072

REVIEW ARTICLES

Cripto: A Tumor Growth Factor and More

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Cripto, a growth factor with an EGF-like domain, and the first member of the EGF-CFC family of genes to be sequenced and characterized, contributes to deregulated growth of cancer cells. A role for Cripto in tumor development has been described in the human and the mouse. Members of the EGF-CFC family are found only in vertebrates: CFC proteins in zebrafish, *Xenopus*, chick, mouse and human have been characterized and indicate some common general functions in development. Cripto expression was first found in human and mouse embryonal carcinoma cells and male teratocarcinomas, and was demonstrated to be over-expressed in breast, cervical, ovarian, gastric, lung, colon, and pancreatic carcinomas in contrast to normal tissues where Cripto expression was invariably low or absent. Cripto may play a role in mammary tumorigenesis, since in vitro, Cripto induces mammary cell proliferation, reduces apoptosis, increases cell migration, and inhibits milk protein expression. This prediction is strengthened by observations of Cripto expression in 80% of human and mouse mammary tumors. At least three important roles for Cripto in development have created considerable interest, and each activity may be distinct in its mechanism of receptor signaling. One role is in the patterning of the anterior–posterior axis of the early embryo, a second is a crucial role in the development of the heart, and a third is in potentiating branching morphogenesis and modulating differentiation in the developing mammary gland. Whether these properties are functions of different forms of Cripto, different Cripto receptors or the distinct domains within this 15–38 kDa glycoprotein are examined here, but much remains to be revealed about this evolutionarily conserved gene product. Since all Cripto receptors have not yet been determined with certainty, future possible uses as therapeutic targets remain to be developed. Cripto is released or shed from expressing cells and may serve as an accessible marker gene in the early to mid-progressive stages of breast and other cancers. Meanwhile some speculations on possible receptor complexes for Cripto signaling in mammary cells are offered here as a spur to further discoveries. J. Cell. Physiol. 190: 267–278, 2002.

CRIPTO IS THE FIRST MEMBER OF A FAMILY OF GENES NOW NAMED EGF-CFC

The Cripto gene (*CR1*), first cloned from a cDNA library derived from a human teratocarcinoma cell line was named accordingly as teratocarcinoma-derived growth factor-1 (*TDGF1* and maps to human chromosome 3p21.3 (Dono et al., 1991; Saccone et al., 1995). Mouse*Tdgf1* (encoding Cripto protein) has been isolated and characterized (Dono et al., 1993; Liguori et al., 1996). Mouse Cripto has 92% similarity to its human counterpart in the EGF-like domain, which is the most conserved of the domains. There is 83% homology in the EGF-like domains between the members of this family and 40% homology in the CFC domain. In all, seven related genes have been cloned, including mouse and human Cryptic (Ciccodicola et al., 1989; Shen et al., 1997; Bamford et al., 2000), *Xenopus* Frl-1(Kinoshita et al., 1995), Zebrafish one-eyed pinhead (*oep*) (Zhang et al., 1998), and chick-Cripto (Colas and Schoenwolf, 2000). The name CFC for the second conserved domain is derived from the combination Cripto/Frl/Cryptic. The sequence, alignments, and functions of the EGF-CFC gene family have been reviewed recently (Salomon et al., 1999, 2000).

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Received 2 October 2001; Accepted 9 November 2001

STRUCTURE OF CRIPTO PROTEIN

Mouse Cripto protein (Cr1) consists of 171 amino acids and unlike the human CR1 (188 amino acids) has a signal sequence, but both proteins can be secreted, #187 (Brandt et al., 1994) and #2384 (Normanno et al., 1995) (Ciccodicola et al., 1989; Dono et al., 1991; Normanno et al., 1994). The protein product of the *TDGF-1* gene in mouse and human will be denoted as Cripto here. The EGF-CFC family of proteins contain several domains (Fig. 1). The signal sequence allows the polypeptide to be processed through the Golgi body and secreted into the medium. In human Cripto, the hydrophobic domain is not large enough to act as a secretory signal, secretion does occur in human cells by an unknown mechanism. The core peptide is ~20 kDa and glycosylation and other post-translational modifications results in a product of Mr = 28–36 kDa. The Cripto produced by vector expression in chinese hamster ovary (CHO) cells is secreted as a 24–28 kDa protein (Brandt et al., 1994). The pattern of six cysteine residues of the EGF-like domain in Cripto is recognizable, but of the three disulfide loops in EGF, loop A is missing and loop B is truncated resulting in a structure that does not bind to the EGFR family of receptors (Bianco et al., 1999). The six cysteine residues of the EGF-like domain form similar disulfide bridges as in EGF, that is 2–4, 1–3, and 5–6 (Lohmeyer et al., 1997). Within the EGF-like domain, the sequence similarity between the seven members is is 60–70%. Next to the EGF-like domain of Cripto, an additional six-cysteine CFC domain is conserved in the analogous proteins at 35–48%. The CFC domain has unspecified, but crucial functions. It also has three disulfide bridges, whose exact disposition is currently unknown but the spacing of the cysteine residues bears some resemblance to the EGF-like domains found in laminin (Abe et al., 1998). The EGF-CFC proteins range from 171 to 202 amino acids with a core protein of 18–21 kDa and an overall sequence identity between these proteins of only 22–32%. Nevertheless, a high level of overlap has been observed in the interspecies developmental activities of family members, as described later. All the members of the EGF-CFC family, except for Oep in Zebrafish, are glycoproteins that contain a single *N*-glycosylation site and potential *O*-glycosylation sites. Recently, a single *O*-linked fucosylation site has been found between the second and third cysteines in the EGF-like domain of all the EGF-CFC proteins (human T88, mouse T72). Loss of this fucosyl residue by mutation of T88 to G, inhibited the ability of Cripto to interact with its cofactor, Nodal, to signal via Smad2 (Schiffer et al., 2001). This rare modification is also present in the EGF-modules of secreted proteins involved in blood coagulation, thus adding to the importance of glycosylation in the functionality of Cripto.

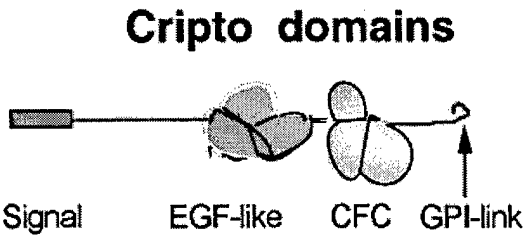

Fig. 1. Cripto protein domains: signal, EGF-like, CFC, and GPI linkage domains.

The hydrophobic domain of Cripto near the carboxy terminus, like other members of the family, has consensus sequences to form a glycerolphosphatidylinositol (GPI) linkage with the cell membrane (Minchiotti et al., 2000). This linkage ensures that secreted Cripto is inserted into the lipids of the plasma membrane perimeter of the cell to form a membrane-anchored extracellular polypeptide, where it could affect cell–cell and cell–matrix interactions. Here, it is likely also to interact with cofactors and receptor polypeptides as well as intracellular linking molecules in lipid rafts, where GPI-linked proteins tend to cluster to effect signaling to the nucleus. A soluble form of Cripto produced by experimental truncation of the carboxy-terminal 16 amino acids to remove the GPI-linkage, is active in growth assays and in null mutation rescue studies in zebrafish embryos (Minchiotti et al., 2001). Human tumors secrete a shortened form of Cripto with an amino-terminal deletion of 42 amino acids by translation from an alternative in frame CUG codon. This protein product likely has activity since it retains the EGF-like and the CFC motif (see below). (For more details on the sequences and roles of the EGF-CFC family of genes, see these reviews: Salomon et al., 1999, 2000.)

EXPRESSION OF CRIPTO IS RESTRICTED

Cripto is expressed early in development

Cripto is expressed early in development in the inner cell mass and the trophoblast cells of the mouse blastocyst (Johnson et al., 1994), in human NTERA2 embryonal carcinoma (EC) cells (Dono et al., 1991; Saccone et al., 1995), in mouse F9 EC cells (Dono et al., 1993; Liguori et al., 1996), and in mouse embryo stem (ES) cells (Xu et al., 1998), but is down-regulated in the first cell types to differentiate from ES cells, namely, visceral and parietal endoderm cells. In the embryo, Cripto is expressed in the E6.5 embryo in the epiblast, primitive streak and ectoplacental cone (Dono et al., 1993; Ding et al., 1998). At E8.5, Cripto is expressed in myocardium of the developing heart tube and in the conotruncus heart outflow region (Dono et al., 1993). Cripto$^{(-/-)}$ embryos die at this stage in utero, by failure to form the anterior–posterior axis and to make sufficient mesoderm, failure to form the primitive streak and to regulate the many genes that center on the activities of the Nodal gene (Ding et al., 1998; Xu et al., 1999; Reissmann et al., 2001; Schier, 2001; Yeo and Whitman, 2001), and see below. Cripto continues to be expressed in the myocardium and outflow region of normal embryos until E10 and then is no longer detected in development. This suggests that Cripto is involved in heart development, but this could not be determined in the Cripto null mouse, because heart-specific Cripto ablation is required for this, a study that awaits completion.

Expression of cripto is restricted after birth

Cripto mRNA is detected at low levels in spleen, testis, heart, lung, and brain (Dono et al., 1991) of the adult mouse, but no function has been described in any normal

tissue except for the mammary gland. Three weeks after birth, the mammary glands of the female mouse start to proliferate from the short ducts at the nipples that developed prenatally. Growth of the ducts becomes active under the influence of pubertal steroid hormones, estrogen, and progesterone. The mammary tree grows rapidly in the virgin into a multi-branched epithelial ductile system to fill the mammary fat pad that limits its further expansion. Cripto is expressed in all epithelial cells at increasing levels during this period. Upon pregnancy, terminal endbuds develop into ductal-alveolar lobules that synthesize milk proteins that enter the duct lumen together with Cripto itself (Bianco et al., 2001). Cripto expression increases during all these stages (Kenney et al., 1995), and starts to decline at the late lactation stage (De Santis et al., 1997) in keeping with the effect of Cripto on mammary epithelial cells described below. When Cripto was experimentally over-expressed in mammary epithelial cells, similar branching structures were formed in vitro and this turned into ductal hyperplasia when Cripto-transduced mammary epithelial cells were transferred to the mouse (Salomon et al., 1999; Wechselberger et al., 2001). It is possible that other tissues such as lung, kidney, and salivary gland form branching structures when stimulated by Cripto expression, but to date none have been reported.

ACTIVITIES OF CRIPTO

Cripto is a growth factor

The growth stimulatory properties of Cripto have been demonstrated on tumor cells of many kinds, pancreatic, ovarian, endometrial, intestinal, and mammary tumor cells. Normal human and mouse mammary epithelial cells also respond to Cripto and are good resources for receptor analysis (see below). The growth responses have been reviewed extensively (Salomon et al., 1999, 2000).

Cripto commonly supports cell survival

The intensity or duration of the Cripto signal or the conditions of treatment (i.e., growing vs. confluent static cells) may account for reports that Cripto stimulates either survival or apoptosis in related cell types CID-9 and HC-11, both derived from mouse mammary COMMA-D cells. These cells have characteristics typical of mid-pregnant mouse mammary gland cells from which they were derived (Medina et al., 1986). When Cripto was expressed constitutively in moderate amounts in CID-9 cells, increased survival was observed. Conversely, cells expressing an antisense (AS) Cripto version of the retroviral vector were retarded in growth and demonstrated increased levels of apoptosis (Niemeyer et al., 1998). Similarly, recombinant Cripto added to growing HC-11 cells stimulated survival (De Santis et al., 1997). However, recombinant Cripto can also induce apoptosis in confluent HC-11 mouse mammary epithelial cells under certain conditions. Culture of confluent cells for two days in the absence of the survival factors, EGF, and insulin, followed by exposure to Cripto caused maximal apoptosis at 3 days (De Santis et al., 2000). These conditions may mimic the regressing or involuting mammary gland, which undergoes apoptosis when lactation must be terminated and the gland returns to a resting condition with reduced size (see below).

Cripto inhibits the differentiation of mammary cells in culture

The synthesis of several milk proteins is stimulated by the culture of cells on a matrix and in the presence of a lactogenic hormone mixture consisting of prolactin, insulin, and dexamethasone (DIP). Using this system, the production of beta-casein and whey acidic protein was inhibited by the over-expression of endogenous Cripto or by the addition of exogenous Cripto to CID-9, HC-11 cells, or primary cultures of mouse mammary epithelial cells established from mid-pregnant mice (De Santis et al., 1997; Niemeyer et al., 1998). These responses agree with the rising expression levels of Cripto in the normal mammary gland during pregnancy and lactation, in parallel with rapid growth of the mammary ductal epithelium and later with milk protein production.

Cripto increases the migration of expressing cells and has a chemotactic effect on other cells

Cripto is over-expressed in cancer cells, and may contribute to metastatic progression by its effect on the shape, adhesivity, and migratory activity of cell. This was shown in CID-9 cells over- or under-expressing Cripto (Niemeyer et al., 1998) and in mouse embryo fibroblasts (MEFs) that were derived from knockout mice. The Cripto null cells attached poorly to fibronectin and collagen, but better to laminin than wild-type MEFs, suggesting that this population of cells could be more epithelial in type. Wild-type MEFs migrated through a porous membrane at a higher rate than $Cr^{(-/-)}$ cells, indicating that the expression of Cripto stimulates cell motility. Conditioned medium from Cripto-secreting cells also attracted higher rates of migration of cells towards it than medium lacking Cripto (Xu et al., 1999). Undoubtedly, these effects contributed to the early lethality (E8–9) of the developing embryo. When cervical carcinoma cells were experimentally induced to over-express Cripto, an increase in cell migration was observed, which correlated with an increase in the expression of vimentin compared to control cells, detected using microarray analyses of mRNA populations in the two cell types (Ebert et al., 2000). Therefore, like many other growth factors, Cripto can affect cell shape and cell migration by secondary effects on the cytoskeleton, as well as by increasing cell proliferation. In embryo cells, the induction of vimentin is indicative of mesodermalization of the epithelial layer and the migration of cells for morphogenesis. In cancer cells, vimentin expression similarly indicates the formation of more mesodermal-like cells with increased migratory activity. Likewise, overexpression of mouse Cripto in mouse mammary EpH4, NOG-8, NMuMG, and TAC-2 epithelial cells showed elevated migration and scattering (Wechselberger et al., 2001).

Cripto is essential for early embryonic development

Homologous recombination of the Cripto gene in ES cells produced normal heterozygous $Cr1^{(+/-)}$ animals. $Cr1^{(-/-)}$ embryos were abnormal on the seventh day of

gestation (E7) and failed to develop further than E9. Embryos failed to form the anterior–posterior axis and while embryonic mesoderm was sparse, extraembryonic mesoderm was present (Ding et al., 1998; Xu et al., 1999). Importantly, chimeric animals made by the injection of $Cr1^{(-/-)}$ ES cells into normal blastocysts were normal and had 50% incorporation of the Cripto null ES cells into all the tissues. Therefore, the wild-type cells were able to rescue the null cells, probably by a combination of juxtacrine and paracrine effects of the anchored and soluble forms of Cripto from the wild-type cells, respectively. This is a good illustration of the effectiveness of soluble Cripto even in embryos where tightly controlled morphogens must be orchestrated.

Cripto is required for cardiogenesis in culture

The development of the heart was shown to require Cripto at an early stage, using ES cells in which Cripto was inactivated by two rounds of targeting to produce $Cr1^{(-/-)}$ ES cells. ES cells normally differentiate as aggregates (embryoid bodies, EBs) to form cardiomyocytes that contract visibly starting at 7 days after the removal of leukemia inhibitory factor (LIF) from the culture medium. Cripto null EBs did not produce contracting cardiomyocytes even during extended culture periods. In these cultures, no cardiac muscle proteins were transcriptionally activated while the transcription factors (Gata4, Nkx2.5, and MEF2) that are known to be required for heart formation were expressed. The re-expression of Cripto sense, but not AS in the Cripto null ES cells rescued the ability of the cells to differentiate into cardiomyocytes, indicating that Cripto is required to activate a set of cardiac genes (Xu et al., 1998). The mechanism of the activity of Cripto in pre-cardiomyocytes is unknown, but these cells will be a good source to explore, for receptors that are specific to this stage of development and for the signaling mechanism.

Cripto plays a role in mammary gland development

Cripto is expressed in the developing mouse mammary gland at increasing levels from 3 weeks of age, under the influence of pubertal hormones. Both mRNA and protein have been observed and immunocytochemical staining showed Cripto protein in all epithelial cell types (Kenney et al., 1995; Niemeyer et al., 1998). Terminal end buds, ductal, and lobular–alveolar cells were all positive and expressed about four-fold higher levels during pregnancy and lactation compared to mature non-pregnant mammary glands. Cripto is released into the milk in the human (Bianco et al., 2001), where it may have some functions as a growth factor. Cripto expression declines rapidly at the end of lactation, and reverts to very low or no expression during involution of the mammary gland. However, in Balb/c mice that reach 2 years of age, the mammary glands re-express Cripto and can develop spontaneous adenocarcinomas (Herrington et al., 1997).

CRIPTO IS AN AUTOCRINE GROWTH FACTOR IN MAMMARY, COLON, PANCREATIC, AND OTHER TUMORS

As indicated above, mouse or human mammary cells over-expressing Cripto exhibit increased proliferation and transformed growth in vitro (Ciardello et al., 1991; Niemeyer et al., 1998). Cripto was detected in human breast cancers, accompanied by members of the EGF ligand family (Panico et al., 1996) and in the early invasive stage of cervical carcinoma (Ertoy et al., 2000). A recent review analyzes the incidence of immunoreactive Cripto in breast, colon, stomach, pancreas, lung, ovary, endometrium, testis, bladder, and prostate tumors (Salomon et al., 2000). The highest significant increase in expression of Cripto compared to non-involved epithelium was in colon, stomach, pancreas, breast, lung, and bladder carcinoma. It was noted that the level of expression increases with degree of dysplasia from pre-malignant to metastatic disease in gastric cancer. Given the common finding of the over-expression of several EGF-related growth factors simultaneously, together with receptor proteins, EGFR (HER1) and ErbB2 (HER2), the additive or synergistic effect may play a significant role in tumorigenesis. Cripto over-expression was shown to repress the expression of E-cadherin and other epithelial markers such that epithelial integrity was compromised. This indicates that Cripto over-expression could be tumorigenic in vivo.

Animal models have been useful in defining molecular interactions in the mammary glands of transgenic mice by over-expressing a transgene from a mammary-specific promoter such as the mouse mammary tumor virus-LTR (MMTV). To determine if Cripto over-expression alone can cause tumor formation, MMTV-CR-1 transgenic mice have been developed (Wechselberger et al., unpublished communications). The results so far show that mammary hyperplasias occur in multiparous females and about 35% of multiparous females develop papillary adenocarcinomas of the mammary gland. In virgin females, there is a dramatic increase in ductal morphogenesis (lateral side branching) and intraductal hyperplasia.

SIMILARITIES OF CRIPTO TO THE FIBROBLAST GROWTH FACTOR (FGF) FAMILY

The similarity of Cripto to FGF is not immediately obvious, but several features are shared. First, the frog *Xenopus laevis* EGF-CFC protein homologue, Frl-1, was cloned from its binding to the *Xenopus* FGF receptor using a functional screen in yeast (Kinoshita et al., 1995). Secondly, both have essential functions in mesoderm and axis formation and patterning in developing embryos. Thirdly, although Cripto does not bind to the FGFR-1 directly, it does have a common docking protein, FGFR substrate-2 (FRS2). FRS2 binds to activated FGFR1 via its phosphotyrosine-binding (PTB) domain, and activates Grb2 and Sos to signal through the RAS/MAPK pathway (Kouhara et al., 1997; Ong et al., 2000). FRS2 is able to attach to the plasma membrane through myristylation, thus bringing it (and the FGFR) close to lipid rafts where Cripto is anchored. Finally, a remarkable finding using three-dimensional

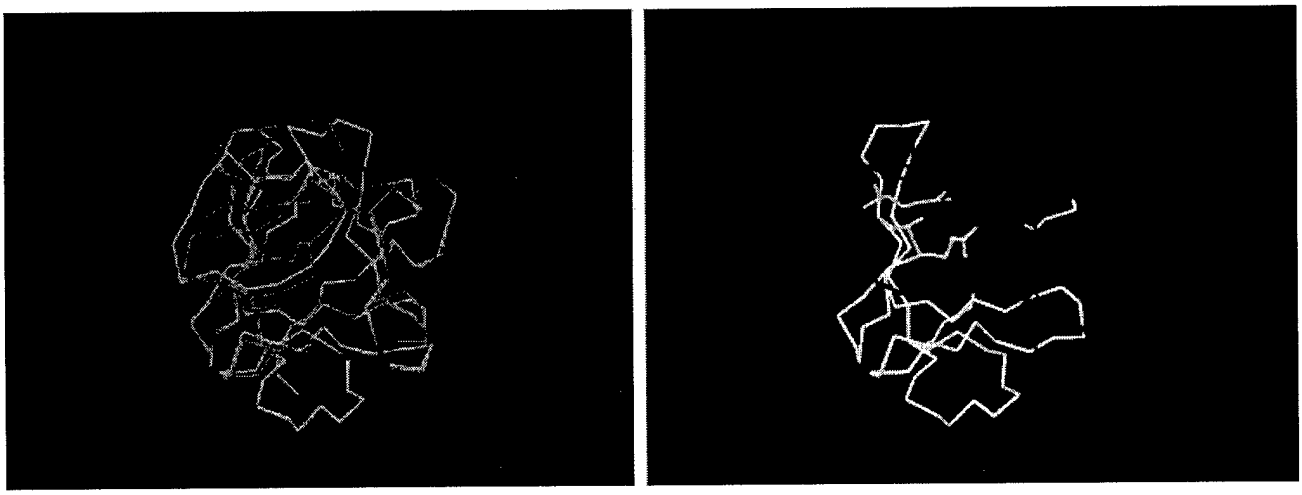

Fig. 2. Molecular structures. **A**: View of a model for Cripto superimposed onto the basic FGF structure (2bfh). The Cα traces are shown, Cripto in magenta; FGF in cyan. **B**: Cripto model with EGF-like domain in red and the CFC domain in blue. Disulfide bonds are shown in yellow. Reproduced from Minchiotti et al. (2001) by permission of the publisher.

protein structure searches produced a very distinct match between the folded structure of Cripto and the β-trefoil structure of FGF2 (Ponting and Russell, 2000; Minchiotti et al., 2001). The overlap in these structures is reproduced in Figure 2A, the resulting predicted structure of Cripto is shown in Figure 2B.

The β-trefoil structure is not restricted to the FGF family, but occurs widely in other factors such as the lymphokine, interleukin 1. A binding domain within this structure may contribute to the ability of FGFs and Cripto to bind to heparin sulfate proteoglycans (HSPG) on the cell surface and in the matrix of the basal lamina. Binding to matrix is thought to enhance the affinity of cell receptors for growth factors held within. These are important clues in predicting how Cripto could have distinct types of receptors for each type of activity (see below).

MECHANISMS UNDERLYING THE ACTIVITIES OF CRIPTO

Signaling pathway

See recent reviews for details of the developmental signaling pathways operating for the EGF-CFC family of ligands (Schier and Shen, 2000; Shen and Schier, 2000; Schier, 2001). Cripto was first assigned to the Epidermal Growth Factor (EGF)-like family of ligands, but the EGF-like domain is unable to bind to any of the EGFR family of receptors. However, it does cross-talk with the ErbB4 receptor and the FGFR-1 indirectly, stimulating the tyrosine phosphorylation of these receptors and contributing to the subsequent stimulation of growth (Bianco et al., 1999; Salomon et al., 1999). The first studies of the receptor in mammary epithelial and A431 cells using recombinant [^{125}I]-Cripto, found no binding to any ErbB receptors. ErbB4 was shown to be activated and tyrosine phosphorylated by Cripto, but not by binding directly. The activation occurred through crosstalk via unknown receptor/adapters, such as c-Src to the ErbB4 receptor. c-Src can activate Shc which then complexes with Grb2 and Sos, for subsequent activation of the Ras/Raf/MEK/MAPK pathway to stimulate transcription factor activity in the nucleus (Kannan et al., 1997). This pathway may explain the proliferative effect of Cripto, but how the signals are generated is still unclear. A further clue to the receptor moieties is that Cripto stimulates the rapid appearance of phosphorylated proteins of 185 kDa (likely ErbB4) and 120, 80, and 60 kDa and recently the possible identity of these intermediaries has been suggested (see below) In addition, PI3 kinase was shown to be activated by Cripto because treatment with the PI3Kinase inhibitor, LY294002 blocked PI3Kinase activation and the inhibitory effect of Cripto on β-casein protein synthesis in HC-11 cells. The data suggest that human Cripto can function as a survival factor through a PI3K-dependent signaling pathway involving AKT and GSK-3β. This pathway could also explain the effects of Cripto on differentiation (De Santis et al., 1997; Ebert et al., 1999).

The interactions of the EGF-CFC proteins in *Xenopus* and Zebrafish have produced good evidence for the receptors that are vital for developmental signaling. In these species, Cripto is required for A/P axis and mesodermal patterning, while Cryptic regulates left–right asymmetry in the heart and other organs. Cripto and Cryptic are represented by one member of the family, Frl-1 in *Xenopus* and Oep in the Zebrafish, *Danio rerio* and they regulate both axis/mesoderm formation and left–right asymmetry. The embryos of these species are readily manipulated to express exogenous mRNA and in some studies, heterologous soluble EGF-CFC proteins have been injected to test their effects on subsequent development. In Zebrafish, strong genetic evidence has indicated that EGF-CFC proteins render cells competent to respond to an instructive signal such as Nodal, or another TGFβ ligand. The receptor for the Oep+Nodal ligands is an Activin-type RIIB that requires an Activin RIB partner to activate the Smad2/Smad3 signaling pathway (Gritsman et al., 1999). For example, recombinant Nodal protein was able to induce Smad2 activation by this pathway in mouse EC (P19)

cells, but only when Cripto was expressed (Kumar et al., 2001).

Receptor reconstitution experiments, have shown that Cripto interacts with ALK4, an Activin Type RIB receptor to permit Nodal binding to the ALK4/Act-IIRB complex, leading to Smad phosphorylation (Yeo and Whitman, 2001). The effect of mutations of single codons in Cripto were tested for their effects as measured by phospho-Smad analysis. Injection of synthetic C-terminus tagged mouse Cripto mRNA into *Xenopus* embryos confirmed the requirement for both the EGF-like and the CFC domains. The Activin receptor ALK4 is able to mediate Nodal signaling, but only in the presence to Cripto; accordingly, recombinant Cripto protein binds directly both Nodal and Alk4 receptor (Reissmann et al., 2001). In *Xenopus* embryo animal caps, the orphan type I serine/threonine kinase receptor ALK7 can also act as a receptor for mouse Nodal and *Xenopus* Nodal-related 1 (Xnr1) in receptor reconstitution experiments, indicating that ALK7 can also interact with ActRIIB to confer responsiveness to Xnr1 and Nodal. Both ALK4 and ALK7 receptors can independently bind Xnr1. In addition, Cripto is implicated in Nodal signaling via ALK7, since its expression enhances the ability of ALK7 and ActRIIB to respond to Nodal ligands and it binds directly Alk7 in co-immunoprecipitation assays (Reissmann et al., 2001). Cripto was found to be required for Nodal signaling to Smad2, but not for Nodal to inhibit Bone Morphogenetic Protein (BMP) signaling (Yeo and Whitman, 2001).

The Zebrafish maternal-zygotic one-eyed pinhead (M*zoep* mutant that lacks EGF-CFC gene expression has defects in anterior–posterior axis, defective trunk formation, as well as L/R asymmetry defects and the presence of a single midline eye. Studies using the mutant animal can be informative by the injection of recombinant protein or mRNA into embryos to determine the degree of rescue of the mutant phenotype. In spite of the species and sequence difference, wild-type mouse Cripto or Xenopus Frl-1 proteins can fully rescue the trunk defects of *oep* mutants and the soluble protein lacking the last 16 amino acids is also active (Gritsman et al., 1999; Minchiotti et al., 2001). Of several mutations in single amino acids in mouse Cripto that were tested, two in the EGF-like domain (G71 and F78) lead to loss of rescue function, defining these residues as essential for Cripto function, at least in this assay. A further four mutations in the EGF-like domain and four more in the CFC-domain gave intermediate rescue in a dose-dependent manner. A structural model that predicts in silico, the molecular shape of Cripto was built (Fig. 2) and shows that the most critical residues are grouped at one side of the molecule. Important residues in the EGF-like (N67, S77, R88, and E91) and CFC domain (H104, L114, L122, and R116) are required, indicating the cooperation of both these motifs in forming an active site (Minchiotti et al., 2001). These data will be useful in testing how Cripto interacts with its putative receptors and whether different receptors are utilized in different tissues.

The question of receptor usage was posed for the effects of Cripto in mammary epithelial cells. Is Nodal, a molecule thought to be restricted to the early embryo, expressed in this tissue? Surprisingly, the answer was affirmative as tested by Bianco et al. (2002). A human brain phage-display library with human recombinant Cripto as bait, was used to detect and identify a receptor in mammary epithelial cells. Phage inserts with identity to the Type I Activin receptor RIB/ALK4, were detected, and shown to bind to and immunoprecipitate with Cripto. No Activin Type II partner for normal Activin receptor activity was found on the surface of mouse mammary cells. However, Nodal was found to be expressed after embryogenesis in the mammary gland and to be required together with ALK4 for Cripto signaling via Smad2 phosphorylation in epithelial cells (Bianco et al., 2002). When recombinant extracellular domains of these Activin receptors were tested in an ELISA binding assay, soluble Cripto bound specifically and with high affinity to the extracellular domain (ECD) of ALK4, but not the ECDs of Activin RIIA or ActivinRIIB. In this in vitro assay, Cripto bound to ALK4 in the absence of any co-receptor. The levels of mRNA expressed in the developing mammary gland measured by RT-PCR, show that the pattern of expression levels of Cripto and Nodal are parallel while ALK4 expression remains unchanged through puberty, pregnancy, and lactation. ActRIIB is also expressed throughout development of the mammary gland with an increase during lactation. Therefore, in the mouse, mammary cells do express the Activin receptor partner to ALK4, even though it does not seem to be required for Cripto binding and signaling by Nodal. Only Nodal, Cripto, and ALK4 are required to generate a signal in a mammary cell line, to activate Smad2. Independent of this signaling pathway, Cripto can also activate the MAPK and the Akt pathways in the same mammary epithelial cells and this does not require Nodal or ALK4. Therefore, the morphogenesis signaling pathway may be generated via Nodal, ALK4, and Smad2, but the proliferative (transforming?) pathway activates MAPK and Akt. This leads to the question of how Cripto finds a receptor without Nodal as a cofactor and of the nature of the receptor. (For a recent review on Activins in organ development, see Ball and Risbridger, 2001.)

CRIPTO RECEPTOR(S)

We hypothesize that the GPI-anchored extracellular form of the Cripto molecule is a crucial characteristic for some of its biological functions based on autocrine effects, ligand recognition, ligand-receptor binding strength and/or efficiency, and possibly also to generate the signals and graded responses by the receptor into the selection of specific signaling pathways. However, the soluble monomer form is also active and must bind to a receptor or a co-receptor at the membrane. The type of Cripto receptor and the affinity of binding are hypothesized to play a large role in the specific signaling pathway used and in the specific response produced in a target cell. There is no evidence that Cripto needs to be activated by dimerization, although this cannot be ruled out once it has bound to a receptor. In general, the more intense the stimulus, the more variable the response may be. For instance, some growth factors including Cripto, can affect cell shape and cell migration by secondary effects on the cytoskeleton, as well as stimulating cell proliferation. This may involve interactions

with the extracellular matrix and these are included in the following models.

There are at least five cellular responses that Cripto can stimulate. They are morphogenesis/differentiation, survival, proliferation, deregulated or transformed growth, and increased motility. The scheme illustrated in Figure 3A offers the simplest combination of molecular components of a small class of proteins that can be assembled at the membrane through the special properties of lipid rafts. Lipid rafts are structures that interrupt the lipid bilayer at the surface of cells by their disordered composition consisting of high levels of cholesterol and sphingolipids. Lipid rafts can form the anchorage for extracellular molecules that have GPI linkages and for intracellular signaling molecules such as c-Src and FRS2, that have myristylated or acylated extensions that enter the lipid raft from inside the cell. The assumption is that once in the raft, they can interact without requiring the protein backbone to be in contact. Model A depicts that Cripto could interact with c-Src, the most common of the non-receptor tyrosine kinases that attach in this way to the membrane. There is good reason to think that c-Src is a partner in this and other schemes, because when Src-like kinases are treated with the inhibitors, PP1 or PP2, downstream activation (i.e., tyrosine transphosphorylation) of ErbB4 and MAPK is blocked in a number of mouse and human mammary epithelial cell lines (Salomon et al., 2000). The data suggest that c-Src is the upstream activator that is directly coupled to Cripto through an ALK-4 and Nodal-independent signaling pathway because ErbB-4 transphosphorylation and MAPK activation are not blocked by a DN-ALK4 in cells that are also null for Nodal expression. Alternatively, in Model B, an unidentified receptor kinase is also inserted into or adjacent to the lipid raft where Cripto can interact without binding directly. Possibly this is how ErbB4 might become phosphorylated. The importance of ErbB4 to signaling in transformed mammary epithelial cells was shown using a ribozyme vector to reduce ErbB4 expression in T47-D human breast cancer cells. MAPK activation by Cripto was severely attenuated in these cells. Whether this is also true for other breast cancer cell lines or other non-transformed mouse or human mammary epithelial cells that also respond to Cripto with respect to MAPK activation is not known.

The signaling pathway utilized by Cripto in the embryo and possibly in the developing mammary gland is probably best represented by Model C in Figure 3. In these tissues, Cripto binds to and requires Nodal, which binds to the Activin Type IB/ALK4 Receptor; probably the p60 kDa protein shown to bind to Cripto (Bianco et al., 1999). In the embryo and mammary gland, the ALK4 receptor partner is ActIIB. The activation of ALK4 leads to the phosphorylation of Smad2 and 3 leading to the activation of Smad4, which then carries the signal to the nucleus for cellular responses by target gene regulation. Model D is a variant of model B, wherein GPI-anchored Cripto, in lipid rafts can interact with myristylated c-Src and with myr-FRS2 that can bind to the FGFR1 when activated. The FGF/FGFR/FRS2 pathway operates in embryos in response to FGF (Ong et al., 2000; Kusakabe et al., 2001) and in the development of the mammary gland. FGFR2 has been shown to play a role in ductal morphogenesis in the virgin mammary gland and in alveolar–lobular development during pregnancy (Jackson et al., 1997) and FGFs and FGFR1 are expressed on breast cancer cells (Yoshimura et al., 1998). Activated FRS2 can bind to Grb2 and to the phosphatase Shp2 to crosstalk with pathways leading to growth and motility. This indirect binding via the FGF signaling pathway is another possible effect of Cripto.

The cytokine receptor gp130 and its partner LIFR are expressed in breast cancer cells (Douglas et al., 1997). LIF, the ligand for this receptor is also expressed in breast cancer cells (Chiu et al., 1996) and has been shown to stimulate the growth of breast cancer cell lines, but with less effect on normal mammary cells (Estrov et al., 1995). It is possible that breast cell growth is, in part, regulated by Cripto interaction with this cytokine system (model E in Fig. 3). Note that the molecular size of gp130 is similar to the size of one of the proteins that is detected after cross-linking to Cripto (Bianco et al., 1999). LIF inhibits the differentiation of ES cells and also induces branching tubulogenesis in the developing kidney (Sariola, 2001), although a similar effect has not been tested in the mammary gland. There is also evidence for a role for LIF and other members of the IL6 family of cytokines in cardiomyocyte growth and function. In ES cell aggregates or EBs differentiating to cardiomyocytes as described earlier, LIF has to be removed for differentiation to start. Since ES cells express Cripto, we speculate that Cripto could bind and replace LIF on the receptor pair, LIFR/gp130 heterodimer depicted in Figure 3E activate the Jak/Stat pathway for differentiation of cardiomyocytes. Consider also that this receptor pair is known to play a role in cardiomyocyte function, for instance, in stress responses leading to cardiomyocyte enlargement and heart failure (Hirota et al., 1999). Another cytokine, cardiotrophin1, also rapidly activates the LIFR/gp130 receptor heterodimer on ES cells and leads to the tyrosine phosphorylation of gp130 in developing cardiomyocytes (Wollert and Chien, 1997). Similarly LIF or IL6 additions to cardiomyocyte cultures cause the cells to stimulate myosin light chain protein (MLC-2) by signaling via the Jak/Stat pathway to increase cardiomyocyte size (Kodama et al., 1997). It is possible, therefore, that Cripto interacts with this receptor system to regulate the development of the cardiomyocyte during embryogenesis. In cancer cells the Stat pathway becomes readily unregulated. Constitutive activation of Stat3 has been detected in a large range of human breast cancer cells and was shown to be maintained by upstream Src and Jak activation and probably generated via activated tyrosine kinase receptors. The application of a dominant negative Stat3 induced apoptosis (Garcia et al., 2001) indicating the importance of the Stat transcription factors in tumorigenesis. Perhaps the activation of the Cripto receptor exacerbates this effect in mammary cancer cells.

Many different cell surface and matrix proteoglycan core proteins are expressed in the mammary gland and in mammary cells in culture. Heparan sulfate proteoglycans at the cell surface modulate the activities of a large number of extracellular ligands such as TGFβ, VEGF, FGF, HGF, and Wnts (Bernfield et al., 1999).

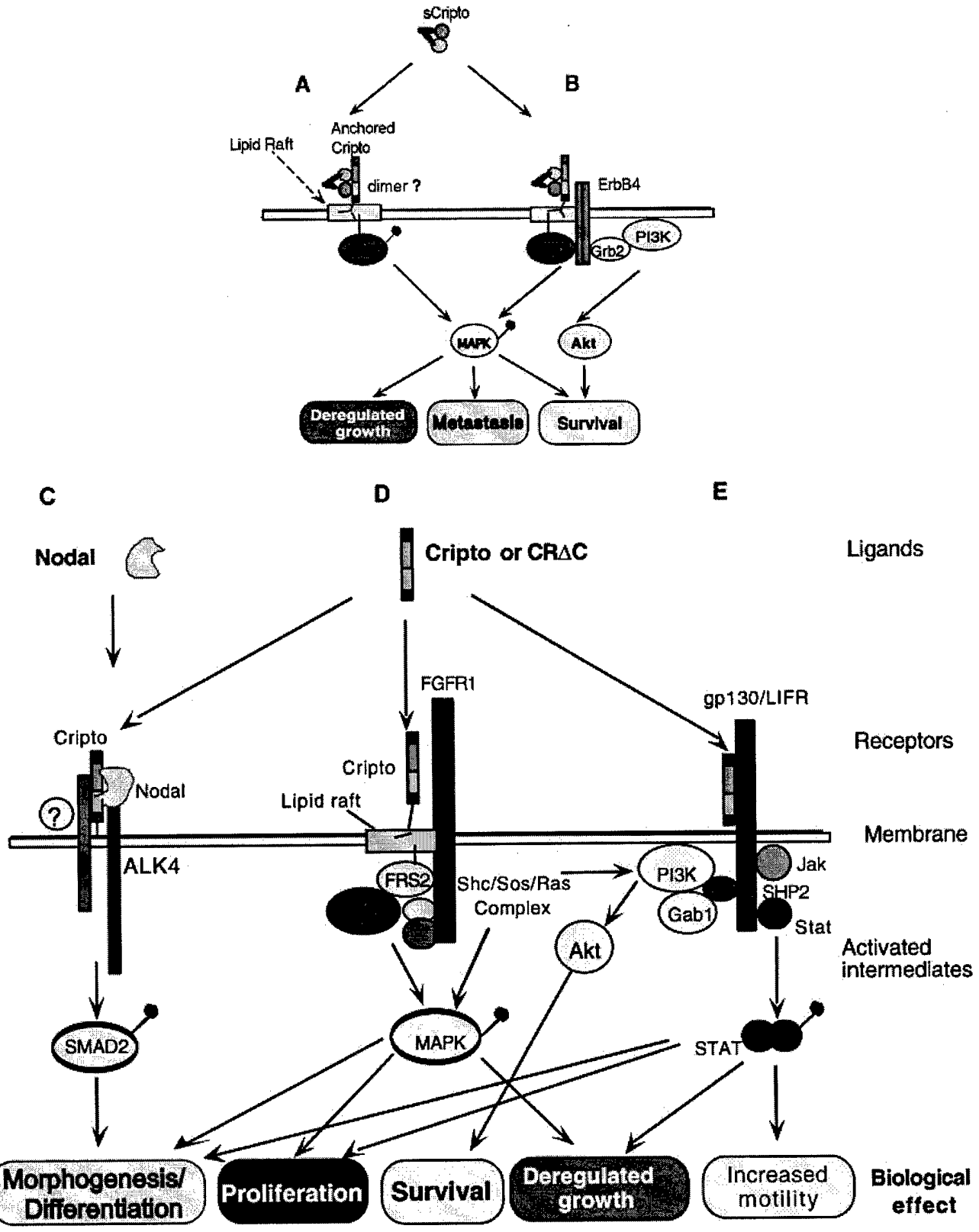

Fig. 3. *(Continued)*

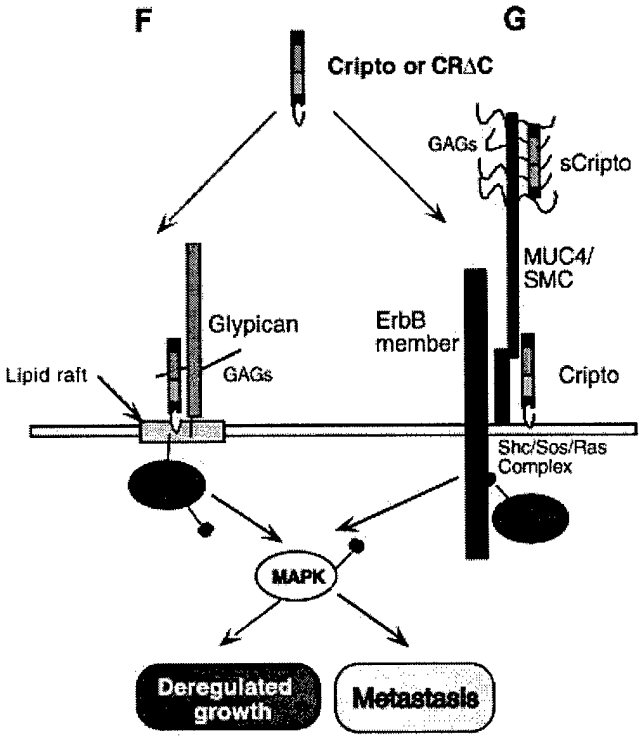

Fig. 3. Putative Cripto receptors and some signaling factors that may operate in the mammary gland. **A**: The simplest mode for Cripto interaction with the intracellular signaling component c-Src, to activate growth, survival, and metastatic pathways. This is possible through the location of the partners in lipid rafts that can bring factors together by their concentration there. **B**: Cripto is known to activate the ErbB4 receptor, but only indirectly. This is known to be important for proliferative responses. **C**: Cripto acts as a cofactor with Nodal to activate the ALK4 (Act-RIB). The involvement of an Act-RIIB does not appear to be necessary as described by Bianco et al. (unpublished communications). **D**: Cripto is known to activate the FGF Receptor-I through the intermediary FRS2, to activate the MAPK pathway. **E**: Cripto could also interact with the gp130/LIFR cytokine receptor complex that is found on ES cells and mammary carcinoma cells, but there is as yet no evidence for this. **F**: Cripto is likely to find another component in lipid rafts, the proteoglycan glypican, whose GAG moieties can bind a number of growth factors. Thus glypican may act as a receptor or co-receptor. **G**: The mucin family of glycoproteins are over-expressed in mammary carcinoma cells and the membrane inserted component of MUC4 could also act as an adapter for crosstalk with the ErbB family.

A molecule of Mr130 kDa is a good candidate for performing a role as a Cripto receptor or co-receptor if it is known to be expressed in a manner that parallels the levels and activities of Cripto. Glypican is a candidate because it is an extracellular proteoglycan of 130 kDa that is GPI inserted into lipid rafts in the membrane (Delehedde et al., 2001) and is known to act as a cofactor in FGF and TGFβ signaling. Glypican is known to bind to some isoforms of VEGF to potentiate binding to the Flk1 receptor and to stimulate angiogenesis (Robinson and Stringer, 2001). Proteoglycans can bind multiple proteins simultaneously and bring them together. Therefore glypican could serve as a cofactor to bind soluble Cripto to promote synergy with other growth factors and their receptors (Fig. 3F) and this could be readily tested. A similar format could operate for the sialomucins (see Fig. 3G for a model). Mucins are a large group of proteins that contain *O*-linked saccharides of which the larger molecules are viscous and generally have a protective function for the cell. Some mucins are membrane inserted and some are soluble. For example, Muc4/Smc is formed from a single polypeptide chain that is proteolytically cleaved into a heterodimer. One subunit, ASGP-2 (sialoglycoprotein) is a membrane inserted protein with two EGF-like domains and is a partner to the larger polypeptide, ASPG-1 of 120–140 kDa, that is heavily decorated with proteoglycan chains (Sherblom and Carraway, 1980). FGF-like proteins such as Cripto can bind to HSPG moieties and potentially generate a signal through the EGF domains in the ASPG-2 chain, which have consensus binding sequences for the ErbB receptors. In this respect, ASGP-2 was shown to co-immunoprecipitate with ErbB2 (Sheng et al., 1992). Interestingly, the expression of Muc4 during mammary development parallels that of Cripto and Muc4, like Cripto, is secreted into the milk. In general Muc4 is over-expressed in cancer cells and inhibits cell–cell interaction, thus contributing to invasiveness and metastatic behavior of cells. A different proteoglycan, DC3/Muc1 is also associated with breast cancer and is able to bind to c-Src after its phosphorylation by the activated EGF receptor (Li et al., 2001). This may also serve to crosstalk with Cripto. (For more details on proteoglycan roles in breast cancer see a recent review Carraway et al., 2001). These suggestions for possible new types of receptors for the Cripto growth factor have some credence and could provide some avenues to explore.

CONCLUSIONS AND POSSIBILITIES FOR CANCER THERAPEUTICS

Cripto as a marker

Cripto could be an important indicator of the initiation of transformation and degree of progression of the tumorigenic process, in carcinoma of the breast, colon, lung, pancreas, stomach, and bladder. Cripto is certainly synthesized at increasing levels with the progression of mammary tumor development in animal models (Kenney et al., 1996; Niemeyer et al., 1999). Cripto has also been shown to cause mammary adenocarcinoma in transgenic mice over-expressing Cripto in mammary tissue.

Cripto activates a number of signaling pathways

Cripto activates a number of signaling pathways of which the most defined is during development. In this case Cripto functions as a cofactor for Nodal, an important morphogen, binding Activin receptor heterodimers and signaling through the Smad transcription factor pathway. Cripto may perform several roles in the mammary gland by stimulating ductal morphogenesis in the virgin animal; by regulating the proliferation of lobular–alveolar epithelial cells during pregnancy; and in the inhibition of the production of milk after lactation and possibly by contributing to apoptosis of the epithelial cells at the end of lactation. High levels of Cripto fall to undetectable levels in the resting gland and only rise again in the event of inappropriate conditions leading to hyperplasia and tumorigenic progression. During the development of the mammary gland, Nodal may be

required with Cripto to interact with the Activin receptors ALK4 or ALK7, to signal via the Smad transcription factors.

Cripto can activate the ErbB4 receptor indirectly

Cripto can activate the ErbB4 receptor indirectly by as yet unknown mechanism(s) and this may be an essential signal during lactation where ErbB4 performs an obligatory role in regulating milk protein expression (Jones et al., 1999), and where c-Src is a required effector in this situation. What is needed now is a search for other receptors that may act through specific mechanisms in specific cell types to mediate Cripto functions.

Other receptors or co-receptors

Receptors that have been found to be over-expressed in tumor cell lines, especially mammary tumor cells and mammary tumors, include ErbB receptors, FGF receptors, the cytokine receptors, proteoglycans glypican and the sialomucins. These components of the normal cell become elevated in breast cancer tissues and could lead to one or all of the putative signaling programs outlined here. In addition, ligands for the ErbB and FGF receptors also become over-expressed to create positive feedback loops. All of these may contribute to increasing progression of growth rates that increase the chances of genetic changes and to inappropriate survival of transformed cells and to the loss of cell adhesion leading to metastases. Each of these avenues is worthy of study.

Attention to some candidate receptors

This review draws attention to some candidate receptors, but more study is needed. Cripto itself or its receptor(s) may be appropriate therapeutic targets in cancer, or more usefully, the prevention of Cripto re-expression or action. In one approach, a ribozyme-based vector was designed that inhibited Cripto translation (Kintner and Hosick, 1998). Likewise, the use of AS oligonucleotides against Cripto have shown activity in inhibiting breast, colon, and ovarian cell growth (Normanno et al., 2001).

Over-expressed growth factors

It should be kept in mind that many other growth factors when over-expressed, are tumorigenic in the mammary gland. Therefore, it is likely that no single growth factor will hold the masterkey to therapy. In this respect a mixture of AS oligonucleotides to Amphiregulin, Cripto and TGFα was shown to inhibit the growth of a human breast carcinoma cell line in vitro, when individually applied at 0.7 μM each (De Luca et al., 1999). The combination of different AS oligos significantly reduced xenograft tumor growth in nude mice treated in vivo, suggesting a possible therapeutic approach (De Luca et al., 2000).

Structure of cripto

Knowledge of the structure of Cripto and its receptors will be a first goal towards intercepting the binding of Cripto and blocking the generation of transforming signals. Since this does not require the agent to enter cells, it is a desirable approach to therapy. Ultimately, only understanding the signal pathway may provide a common therapeutic target that may be a more general focus for intervention. If the hypothesis is correct that Cripto operates through several signaling pathways, each leading to a distinct response, then it will be possible to target tumor cells specifically.

LITERATURE CITED

Abe Y, Odaka M, Inagaki F, Lax I, Schlessinger J, Kohda D. 1998. Disulfide bond structure of human epidermal growth factor receptor. J Biol Chem 273:11150–11157.

Ball EMA, Risbridger GP. 2001. Activins as regulators of branching morphogenesis [review]. Dev Biol 238:1–12.

Bamford RN, Roessler E, Burdine RD, Saplakoglu U, dela Cruz J, Splitt M, Towbin J, Bowers P, Marino B, Schier AF, Shen MM, Muenke M, Casey B. 2000. Loss-of-function mutations in the EGF-CFC gene CFC1 are associated with human left–right laterality defects. Nat Genet 26:365–369.

Bernfield M, Gotte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J, Zako M. 1999. Functions of cell surface heparan sulfate proteoglycans. Annu Rev Biochem 68:729–777.

Bianco C, Kannan S, De Santis M, Seno M, Tang CK, Martinez-Lacaci I, Kim N, Wallace-Jones B, Lippman ME, Ebert AD, Weehselberger C, Salomon DS. 1999. Cripto-1 indirectly stimulates the tyrosine phosphorylation of erb B-4 through a novel receptor. J Biol Chem 274:8624–8629.

Bianco C, Wechselberger C, Ebert A, Khan NI, Sun YP, Salomon DS. 2001. Identification of Cripto-1 in human milk. Breast Cancer Res Treat 66:1–7.

Bianco C, Adkins H, Wechselberger C, Seno M, Normanno N, De Luca A, Sun Y, Khan N, Kenney N, Williams K, Sanicola M, Salomon DS. 2002. Cripto-1 activates Nodal and ALK4 dependent and independent signalling pathways in mammary epithelial cells. (In press).

Brandt R, Normanno N, Gullick WJ, Lin JH, Harkins R, Scheider D, Jones BW, Ciardiello F, Persico MG, Armenante F, Kim N, Salomon DS. 1994. Identification and biological characterization of an epidermal growth factor-related protein: Cripto-1. J Biol Chem 269:17320–17328.

Carraway KL, Price-Schiavi SA, Komatsu M, Jepson S, Perez A, Carraway CA. 2001. Muc4/sialomucin complex in the mammary gland and breast cancer. J Mammary Gland Biol Neoplasia 6:323–337.

Chiu JJ, Sgagias MK, Cowan KH. 1996. Interleukin 6 acts as a paracrine growth factor in human mammary carcinoma cell lines. Clin Cancer Res 2:215–221.

Ciardello F, Dono R, Kim N, Persico MG, Salomon DS. 1991. Expression of *cripto*, a novel gene of the epidermal growth factor gene family, leads to in vitro transformation of a normal mouse mammary epithelium cell line. Cancer Res 51:1051–1054.

Ciccodicola A, Dono R, Obici S, Simeone A, Zollo M, Persico MG. 1989. Molecular characterization of a gene of the 'EGF family' expressed in undifferentiated human NTERA2 teratocarcinoma cells. EMBO J 8:1987–1991.

Colas JF, Schoenwolf GC. 2000. Subtractive hybridization identifies chick-cripto, a novel EGF-CFC ortholog expressed during gastrulation, neurulation and early cardiogenesis. Gene 255:205–217.

De Luca A, Casamassimi A, Selvam MP, Losito S, Ciardiello F, Agrawal S, Salomon DS, Normanno N. 1999. EGF-related peptides are involved in the proliferation and survival of MDA-MB-468 human breast carcinoma cells. Int J Cancer 80:589–594.

De Luca A, Arra C, D'Antonio A, Casamassimi A, Losito S, Ferraro P, Ciardiello F, Salomon DS, Normanno N. 2000. Simultaneous blockage of different EGF-like growth factors results in efficient growth inhibition of human colon carcinoma xenografts. Oncogene 19:5863–5871.

De Santis ML, Kannan S, Smith GH, Seno M, Bianco C, Kim N, Martinez-Lacaci I, Wallace-Jones B, Salomon DS. 1997. Cripto-1 inhibits beta-casein expression in mammary epithelial cells through a p21ras-and phosphatidylinositol 3′-kinase-dependent pathway. Cell Growth Differ 8:1257–1266.

De Santis ML, Martinez-Lacaci I, Bianco C, Seno M, Wallace-Jones B, Kim N, Ebert A, Wechselberger C, Salomon DS. 2000. Cripto-1 induces apoptosis in HC-11 mouse mammary epithelial cells. Cell Death Differ 7:189–196.

Delehedde M, Lyon M, Sergeant N, Rahmoune H, Fernig DG. 2001. Proteoglycans: Pericellular and cell surface multireceptors that integrate external stimuli in the mammary gland. J Mammary Gland Biol Neoplasia 6:253–273.

Ding J, Yang Y, Yan Y-T, Chan A, Desai N, Wynshaw-Boris A, Shen MM. 1998. Cripto is required for correct orientation of the anterior–posterior axis in the mouse embryo. Nature 395:702–707.

Dono R, Montuori N, Rocchi M, De Ponti-Zilli L, Ciccodicola A, Persico MG. 1991. Isolation and characterization of the CRIPTO autosomal gene and its X-linked related sequence. Am J Hum Genet 49:555–565.

Dono R, Scalera L, Pacifico F, Acampora D, Persico MG, Simeone A. 1993. The murine cripto gene: Expression during mesoderm induction and early heart morphogenesis. Development 118:1157–1168.

Douglas AM, Goss GA, Sutherland RL, Hilton DJ, Berndt MC, Nicola NA, Begley CG. 1997. Expression and function of members of the cytokine receptor superfamily on breast cancer cells. Oncogene 14:661–669.

Ebert AD, Wechselberger C, Frank S, Wallace-Jones B, Seno M, Martinez-Lacaci I, Bianco C, De Santis M, Weitzel HK, Salomon DS. 1999. Cripto-1 induces phosphatidylinositol 3′-kinase-dependent phosphorylation of AKT and glycogen synthase kinase 3 beta in human cervical carcinoma cells. Cancer Res 59:4502–4505.

Ebert AD, Wechselberger C, Nees M, Clair T, Schaller G, Martinez-Lacaci I, Wallace-Jones B, Bianco C, Weitzel HK, Salomon DS. 2000. Cripto-1-induced increase in vimentin expression is associated with enhanced migration of human Caski cervical carcinoma cells. Exp Cell Res 257:223–229.

Ertoy D, Ayhan A, Sarac E, Karaagaoglu E, Yasui W, Tahara E. 2000. Clinicopathological implication of cripto expression in early stage invasive cervical carcinomas. Eur J Cancer 36:1002–1007.

Estrov Z, Samal B, Lapushin R, Kellokumpu-Lehtinen P, Sahin AA, Kurzrock R, Talpaz M, Aggarwal BB. 1995. Leukemia inhibitory factor binds to human breast cancer cells and stimulates their proliferation. J Interferon Cytokine Res 15:905–913.

Garcia R, Bowman TL, Niu G, Yu H, Minton S, Muro-Cacho CA, Cox CE, Falcone R, Fairclough R, Parsons S, Laudano A, Gazit A, Levitzki A, Kraker A, Jove R. 2001. Constitutive activation of Stat3 by the Src and JAK tyrosine kinases participates in growth regulation of human breast carcinoma cells. Oncogene 20:2499–2513.

Gritsman K, Zhang J, Cheng S, Heckscher E, Talbot WS, Schier AF. 1999. The EGF-CFC protein one-eyed pinhead is essential for nodal signaling. Cell 97:121–132.

Herrington EE, Ram TG, Salomon DS, Johnson GR, Gullick WJ, Kenney N, Hosick HL. 1997. Expression of epidermal growth factor-related proteins in the aged adult mouse mammary gland and their relationship to tumorigenesis. J Cell Physiol 170:47–56.

Hirota H, Chen J, Betz UAK, Rajewsky K, Gu Y, Ross J, Muller W, Chien KR. 1999. Loss of a gp130 cardiac muscle cell survival pathway is a critical event in the onset of heart failure during biomechanical stress. Cell 97:189–198.

Jackson D, Bresnick J, Rosewell I, Crafton T, Poulsom R, Stamp G, Dickson C. 1997. Fibroblast growth factor receptor signalling has a role in lobuloalveolar development of the mammary gland. J Cell Sci 110:1261–1268.

Johnson SE, Rothstein JL, Knowles BB. 1994. Expression of epidermal growth factor family gene members in early mouse development. Dev Dyn 201:216–226.

Jones FE, Welte T, Fu XY, Stern DF. 1999. ErbB4 signaling in the mammary gland is required for lobuloalveolar development and Stat5 activation during lactation. J Cell Biol 147:77–88.

Kannan S, De Santis M, Lohmeyer M, Riese DJ, Smith GH, Hynes N, Seno M, Brandt R, Bianco C, Persico G, Kenney N, Normanno N, Martinez-Lacaci I, Ciardello F, Stern DF, Gullick WJ, Salomon DS. 1997. Cripto enhances the tyrosine phosphorylation of Shc and activates mitogen-activated protein kinase (MAPK) in mammary epithelial cells. J Biol Chem 272:3330–3335.

Kenney NJ, Huang RP, Johnson GR, Wu JX, Okamura D, Matheny W, Kordon E, Gullick WJ, Plowman G, Smith GH, Salomon DS, Adamson ED. 1995. Detection and location of amphiregulin and Cripto-1 expression in the developing postnatal mouse mammary gland. Mol Reprod Dev 41:277–286.

Kenney NJ, Smith GH, Maroulakou IG, Green JH, Muller WJ, Callahan R, Salomon DS, Dickson RB. 1996. Detection of amphiregulin and Cripto-1 in mammary tumors from transgenic mice. Mol Carcinog 15:44–56.

Kinoshita N, Minshull J, Kirschner MW. 1995. The identification of two novel ligands of the FGF receptor by a yeast screening method and their activity in Xenopus development. Cell 83:621–630.

Kintner RL, Hosick HL. 1998. Reduction of Cripto-1 expression by a hammerhead-shaped RNA molecule results from inhibition of translation rather than mRNA cleavage. Biochem Biophys Res Commun 245:774–779.

Kodama H, Fukuda K, Pan J, Makino S, Baba A, Hori S, Ogawa S. 1997. Leukemia inhibitor factor, a potent cardiac hypertrophic cytokine, activates the JAK/STAT pathway in rat cardiomyocytes. Circ Res 81:656–663.

Kouhara H, Hadari YR, Spivak-Kroizman T, Schilling J, Bar-Sagi D, Lax I, Schlessinger J. 1997. A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. Cell 89:693–702.

Kumar A, Novoselov V, Celeste AJ, Wolfman NM, ten Dijke P, Kuehn MR. 2001. Nodal signaling uses activin and transforming growth factor-beta receptor-regulated Smads. J Biol Chem 276:656–661.

Kusakabe M, Masuyama N, Hanafusa H, Nishida E. 2001. Xenopus FRS2 is involved in early embryogenesis in cooperation with the Src family kinase Laloo. EMBO Rep 2:727–735.

Li Y, Ren J, Yu W, Li Q, Kuwahara H, Yin L, Carraway KL 3rd, Kufe D. 2001. The epidermal growth factor receptor regulates interaction of the human DF3/MUC1 carcinoma antigen with c-Src and beta-catenin. J Biol Chem 276:35239–35242.

Liguori G, Tucci M, Montuori N, Dono R, Lago CT, Pacifico F, Armenante F, Persico MG. 1996. Characterization of the mouse Tdgf1 gene and Tdgf pseudogenes. Mamm Genome 7:344–348.

Lohmeyer M, Harrison PM, Kannan S, DeSantis M, O'Reilly NJ, Sternberg MJ, Salomon DS, Gullick WJ. 1997. Chemical synthesis, structural modeling, and biological activity of the epidermal growth factor-like domain of human cripto. Biochemistry 36:3837–3845.

Medina D, Oborn CJ, Kittrell FS, Ullrich RL. 1986. Properties of mouse mammary epithelial cell lines characterized by in vivo transplantation and in vitro immunocytochemical methods. J Natl Cancer Inst 76:1143–1156.

Minchiotti G, Parisi S, Liguori G, Signore M, Lania G, Adamson ED, Lago CT, Persico GM. 2000. Membrane-anchorage of Cripto protein by glycosylphosphatidylinositol and its distribution during early mouse development. Mech Dev 90:133–142.

Minchiotti G, Manco G, Parisi S, Lago C, Rosa F, Persico G. 2001. Structure-function analysis of the EGF-CFC family member Cripto identifies residues essential for nodal signalling. Development 128:4501–4510.

Niemeyer CC, Persico MG, Adamson ED. 1998. Cripto: Roles in mammary cell growth, survival, differentiation and transformation. Cell Death Differ 5:440–449.

Niemeyer CC, Spencer-Dene B, Wu J-X, Adamson ED. 1999. Preneoplastic mammary tumor markers: Cripto and Amphiregulin are overexpressed in hyperplastic stages of tumor progression in transgenic mice. Int J Cancer 81:588–591.

Normanno N, Ciardiello F, Brandt R, Salomon DS. 1994. Epidermal growth factor-related peptides in the pathogenesis of human breast cancer. Breast Cancer Res Treat 29:11–27.

Normanno N, Kim N, Wen D, Smith K, Harris AL, Plowman G, Colletta G, Ciardiello F, Salomon DS. 1995. Expression of messenger RNA for amphiregulin, heregulin, and cripto-1, three new members of the epidermal growth factor family, in human breast carcinomas. Breast Cancer Res Treat 35:293–297.

Normanno N, Bianco C, De Luca A, Salomon DS. 2001. The role of EGF-related peptides in tumor growth. Front Biosci 6:D685–D707.

Ong SH, Guy GR, Hadari YR, Laks S, Gotoh N, Schlessinger J, Lax I. 2000. FRS2 proteins recruit intracellular signaling pathways by binding to diverse targets on fibroblast growth factor and nerve growth factor receptors. Mol Cell Biol 20:979–989.

Panico L, D'Antonio A, Salvatore G, Mezza E, Tortora G, De Laurentiis M, De Placido S, Giordano T, Merino M, Salomon DS, Mullick WJ, Pettinato G, Schnitt SJ, Bianco AR, Ciardiello F. 1996. Differential immunohistochemical detection of transforming growth factor alpha, amphiregulin and CRIPTO in human normal and malignant breast tissues. Int J Cancer 65:51–56.

Ponting CP, Russell RB. 2000. Identification of distant homologues of fibroblast growth factors suggests a common ancestor for all beta-trefoil proteins. J Mol Biol 302:1041–1047.

Reissmann E, Jornvall H, Blokzijl A, Andersson O, Chang C, Minchiotti G, Persico MG, Ibanez CF, Brivanlou AH. 2001. The orphan receptor ALK7 and the Activin receptor ALK4 mediate signaling by Nodal proteins during vertebrate development. Genes Dev 15:2010–2022.

Robinson CJ, Stringer SE. 2001. The splice variants of vascular endothelial growth factor (VEGF) and their receptors. J Cell Sci 114:853–865.

Saccone S, Rapisarda A, Motta S, Dono R, Persico GM, Della Valle G. 1995. Regional localization of the human EGF-like growth factor

CRIPTO gene (TDGF-1) to chromosome 3p21. Hum Genet 95:229–230.

Salomon DS, Bianco C, De Santis M. 1999. Cripto: A novel epidermal growth factor (EGF)-related peptide in mammary gland development and neoplasia [review]. Bioessays 21(1):61–70.

Salomon DS, Bianco C, Ebert AD, Khan NI, De Santis M, Normanno N, Wechselberger C, Seno M, Williams K, Sanicola M, Foley S, Gullick WJ, Persico G. 2000. The EGF-CFC family: Novel epidermal growth factor-related proteins in development and cancer. Endocr Relat Cancer 7:199–226.

Sariola H. 2001. The neurotrophic factors in non-neuronal tissues. Cell Mol Life Sci 58:1061–1066.

Schier AF. 2001. Axis formation and patterning in zebrafish. Curr Opin Genet Dev 11:393–404.

Schier AF, Shen MM. 2000. Nodal signalling in vertebrate development. Nature 403:385–389.

Schiffer SG, Foley S, Kaffashan A, Hronowski X, Zichittella A, Yeo CY, Miatkowski K, Adkins HB, Damon B, Whitman M, Sanicola M, Williams KP. 2001. Fucosylation of Cripto is required for its ability to facilitate Nodal signaling. J Biol Chem 276:37769–37778.

Shen MM, Schier AF. 2000. The EGF-CFC gene family in vertebrate development. Trends Genet 16:303–309.

Shen MM, Wang H, Leder P. 1997. A differential display strategy identifies Cryptic, a novel EGF-related gene expressed in the axial and lateral mesoderm during mouse gastrulation. Development 124:429–442.

Sheng Z, Wu K, Carraway KL, Fregien N. 1992. Molecular cloning of the transmembrane component of the 13762 mammary adenocarcinoma sialomucin complex. A new member of the epidermal growth factor superfamily. J Biol Chem 267:16341–16346.

Sherblom AP, Carraway KL. 1980. A complex of two cell surface glycoproteins from ascites mammary adenocarcinoma cells. J Biol Chem 255:12051–12059.

Wechselberger C, Ebert AD, Bianco C, Khan NI, Sun Y, Wallace-Jones B, Montesano R, Salomon DS. 2001. Cripto-1 enhances migration and branching morphogenesis of mouse mammary epithelial cells. Exp Cell Res 266:95–105.

Wollert KC, Chien KR. 1997. Cardiotrophin-1 and the role of gp130-dependent signaling pathways in cardiac growth and development [review] [98 refs]. J Mol Med 75(7):492–501.

Xu C, Liguori G, Adamson ED, Persico MG. 1998. Cardiac differentiation is blocked in embryonic stem cells lacking Cripto-1. Dev Biol 196:237–247.

Xu C, Liguori G, Persico MG, Adamson ED. 1999. Abrogation of the Cripto gene in mouse leads to failure of post-gastrulation morphogensis and lack of differentiation of cardiomyocytes. Development 126:483–494.

Yeo C, Whitman M. 2001. Nodal signals to Smads through Cripto-dependent and Cripto-independent mechanisms. Mol Cell 7:949–957.

Yoshimura N, Sano H, Hashiramoto A, Yamada R, Nakajima H, Kondo M, Oka T. 1998. The expression and localization of fibroblast growth factor-1 (FGF-1) and FGF receptor-1 (FGFR-1) in human breast cancer. Clin Immunol Immunopathol 89:28–34.

Zhang J, Talbot WS, Schier AF. 1998. Positional cloning identifies zebrafish one-eyed pinhead as a permissive EGF-related ligand required during gastrulation. Cell 92:241–251.